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Mechanisms of Asbestos-Induced Squamous Metaplasia in Tracheobronchial Epithelial Cells

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Within 1 to 4 weeks after exposure to asbestos, differentiated rodent and human tracheobronchial epithelial cells in organ culture undergo squamous metaplasia, a putative preneoplastic lesion characterized by conversion of mucociliary cell types to keratinizing cells. The exogenous addition of retinal acetate (RA) to culture medium of hamster tracheal organ cultures reverses preestablished, asbestos-induced squamous metaplasia, although data suggest that the effectiveness of RA decreases as the length of time between exposure to asbestos and initial application of RA increases.

α-Difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), inhibits squamous metaplasia caused by asbestos or vitamin A deficiency, whereas addition of methylglyoxal bis(guanylhydrazone) (MGBG), a structural analog of spermidine and inhibitor of S-adenosylmethionine decarboxylase, causes an enhancement of metaplasia under both circumstances. Basal cell hyperplasia and increased incorporation of ^{3}H-thymidine by tracheal epithelial cells also are seen after addition of the polyamines, putrescine or spermidine, to tracheal organ cultures, an observation supporting the importance of polyamines in the development of this lesion. The use of retinoids and inhibitors of ODC could be promising as preventive and/or therapeutic approaches for individuals at high risk for development of asbestos-associated diseases.

Introduction

"Asbestos" refers to a family of hydrated silicates of fibrous (> 3:1 length:diameter ratio) dimensions. Occupational exposure to these minerals has been linked to the development of pulmonary fibrosis (asbestosis), mesothelioma, and lung cancer (i.e., bronchogenic carcinoma) (*1*). The latter disease is of critical importance as it has an extremely poor prognosis and is the cancer type associated with the highest mortality rate in man.

Both epidemiologic and experimental data suggest that asbestos is a cocarcinogen and/or tumor promoter in the development of bronchogenic carcinoma (*2*). For example, in comparison to smokers in the general population (8- to 10-fold increased risk of bronchogenic carcinoma), nonsmoking asbestos workers have a 1.5- to 4-fold increased risk of lung cancer. In contrast, asbestos workers who smoke have a more striking risk (80- to 92-fold) of disease (*3*). With the exception of the rat, most animals do not develop bronchogenic carcinoma after inhalation or intratracheal instillation of asbestos unless polycyclic aromatic hydrocarbons (PAH), chemical carcinogens in cigarette smoke, are adsorbed to the surfaces of the fibers (*4*). Thus, asbestos appears to act as a cocarcinogen by delivering PAH to tracheobronchial epithelial cells (*5*), the progenitor cells of bronchogenic carcinoma.

Tumor promotion by asbestos has been demonstrated in rat tracheal grafts exposed previously to noncarcinogenic amounts of the PAH, dimethylbenzanthracene (*6*). Subsequent insertion of asbestos into these grafts causes the development of tumors, whereas neoplasms are not observed after application of identical concentrations of DMBA or asbestos alone. To elucidate possible mechanisms of asbestos-induced tumor promotion in the respiratory tract, work in this laboratory has focused on the biologic effects of asbestos on hamster tracheal epithelial cells in monolayer and organ culture. Many of the changes reported in cultured cells exposed to phorbol esters, classical tumor promoters studied extensively in mouse skin, are observed in tracheal epithelium after addition of as-

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bestos. These include stimulation of plasma membrane marker enzymes (*7*), increased cell division (*8,9*), increased activity of ODC (*9*), and development of hyperplastic and metaplastic changes (*8,10–12*).

Understanding the pathogenesis of squamous metaplasia is of particular relevance to the development of bronchogenic carcinoma, as the lesion is considered an intermediate step in the progression of morphologic events leading to neoplasia. Although squamous metaplasia associated with trauma or vitamin A deficiency is reversible, it is unclear whether metaplastic changes caused by chemical or physical carcinogens, such as asbestos, resolve with time or develop into malignancies. For example, squamous metaplasia is observed commonly in the respiratory tract of smokers, a group at high risk of developing bronchogenic carcinoma (*13*).

The results of studies from this laboratory suggest that asbestos-induced squamous metaplasia occurs when fibers impinge on the tracheal epithelium (*8,10–12*). Fibers then cause sloughing of superficial cells and compensatory regeneration of epithelial cells that are squamous in nature. Whereas smaller fibers are phagocytized successfully by macrophages and epithelial cells, longer fibers appear to act as matrices for proliferation of cells over their surfaces. Thus, asbestos-induced metaplastic lesions occur at localized sites of accumulation of fibers on the epithelial surface, unlike the broad expanses of metaplasia observed in vitamin A deficiency (*14*).

Work here was initiated to determine if retinoids, i.e., synthetic derivatives of vitamin A, could reverse preestablished squamous metaplasia in hamster tracheal organ cultures exposed to crocidolite asbestos or the PAH, benzo(a)pyrene (BaP). Because retinoids appear to influence polyamine and DNA synthesis (*15*), we also investigated the ability of various inhibitors of polyamine biosynthesis to modify squamous metaplasia caused by vitamin A deficiency or exposure to asbestos. Last, the polyamines putrescine, spermine, and spermidine were added to tracheal organ cultures to determine if they caused increased DNA synthesis, as measured by incorporation of ^{3}H-thymidine, in tracheal epithelium.

Materials and Methods

Preparation of Tracheal Organ Cultures

The technique for preparation and culture hamster tracheal explants has been described in detail previously (*16*). In brief, female golden Syrian hamsters (6–8 weeks of age) were sacrificed by IP injection of sodium pentobarbital, and the tracheas dissected and cleaned of surrounding tissue. After the tracheas were opened longitudinally, they were cut again in half and sectioned into double ring explants. Tissues were divided into groups and cultured in 35-mm plastic culture dishes containing 4 to 5 explants per dish. The explants were maintained in 0.5 mL serum-free Minimum Essential Medium (MEM) (GIBCO) supplemented with 100 μg/mL gentamycin and 25 units/mL nystatin (*16*). Cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 and the culture medium changed three times per week.

Reversion of Squamous Metaplasia

Tracheal organ cultures were divided equally into two groups with three treatment regimens (A, B, and C) as indicated in Figure 1. Each group contained untreated tracheas (A); explants exposed to benzo[a]pyrene (BaP) (0.5 μg/mL medium, dissolved in acetone at a final concentration of 0.1% in medium) three times per week for 3 weeks (B); and tissues exposed to crocidolite asbestos (UICC reference sample) (4 μg/mL medium) for 1 hr at the time of initiation of cultures (C) (*6,8,9,11*). Each of the treatment groups was further subdivided with the subdivisions receiving no retinal acetate (RA), or RA (Sigma Chemical Company) (dissolved in dimethyl sulfoxide at a final concentration of 0.1% in medium) at 10^{-7} M or 10^{-8} M three times per week for 1 week.

Groups 1 and 2 received the RA at different times. Whereas group 1 received the RA for 1 week at week 3 of culturing (T_0), group 2 received the RA for 1 week at 5 weeks (T_1). All explants were harvested at the end of the RA treatments.

Prevention of Squamous Metaplasia

Hamster tracheal organ cultures were prepared as described and divided into groups (n = 9–15 explants/group). To determine whether inhibitors of polyamine biosynthesis affected squamous metaplasia caused by asbestos (protocol #1), untreated controls and explants exposed initially for 1 hr to crocidolite asbestos (4 mg/mL medium) were maintained in MEM with and without addition of DFMO (5 mM, Merrell National Labs) or MGBG (5 μM, Merrell National Labs). Medium with and without drugs was replenished three times weekly. An additional group received DFMO (5 mM) followed at 24 hr by MGBG (5 μM) three times weekly.

In other experiments, nonasbestos-exposed cultures were maintained in Waymouth's MAB 87/3 medium (GIBCO) with the addition of insulin (1 μg/mL medium), hydrocortisone (0.1 μg/mL medium), and antibiotics. This formulation results in a complex vitamin A-deficient medium causing squamous metaplasia (*16*). DFMO (1 or 5 mM), MGBG (5 μm), or DFMO (1 μm) followed by MGBG (5 μm) at 24 hr was added to designated cultures three times weekly (protocol #2).

Autoradiographic Studies

We reported previously (*17*) an increase in the extent of squamous metaplasia in hamster tracheal organ cultures exposed to putrescine (1 mM) in culture medium. To determine if addition of polyamines would increase basal cell hyperplasia as measured by incorporation of ^{3}H-thymidine in tracheal epithelium, tracheal organ cultures were prepared as described above and maintained in Waymouth's MAB/873 with additives. The medium in selected groups then was supplemented with putrescine, spermidine, or spermine (all at 1 and 10 mM; Sigma Chemical Company) three times weekly for 3 weeks. At this time, organ cultures (n = 14–19/group) were pulsed for 5 hr with ^{3}H-thymidine (10 μCi/mL medium) (New England

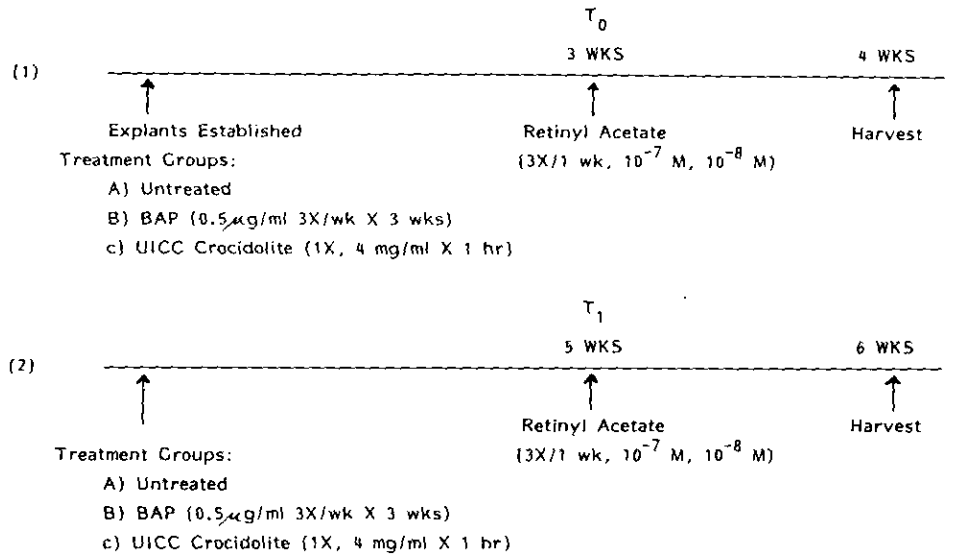


FIGURE 1. Protocols for evaluation of whether retinal acetate reverses squamous metaplasia in control (*A*), BaP-exposed (*B*), and asbestos-exposed (*C*) hamster tracheal organ cultures.

Nuclear) before preparation for histology as described below. Unstained 5 μm sections were prepared for autoradiography as described previously (*8*) and assessed by light microscopy for numbers of epithelial cells incorporating ^{3}H-thymidine.

Histology and Grading of Squamous Metaplasia

All organ cultures were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The grading system for scoring the extent of metaplasia has been reported previously (*8*). Briefly, the prepared sections were examined by light microscopy and given a score depending on the extent of metaplasia observed. Cultures showing normal differentiation and no metaplasia were given a score of 1. Explants with focal metaplastic lesions that covered less than 15% of the epithelial surface were considered as a 2. If the metaplasia covered more than 15% but less than 50% of the epithelium, the explant was scored as a 3. Explants with metaplasia covering more than 50% of the epithelium were scored as 4s.

Slides were coded and scored independently by two investigators, and the scores were averaged. In studies using RA to inhibit squamous metaplasia, data were analyzed by a multiway analysis of variance with the metaplastic score treated as the dependent variable and the other factors (dosage, time, and treatment) adjusted for in the analysis (*18*). The Kruskal-Wallis analysis was used to evaluate metaplastic changes in organ cultures exposed to polyamines and inhibitors of polyamine biosynthesis (*18*).

Results and Discussion

Effects of RA on Squamous Metaplasia

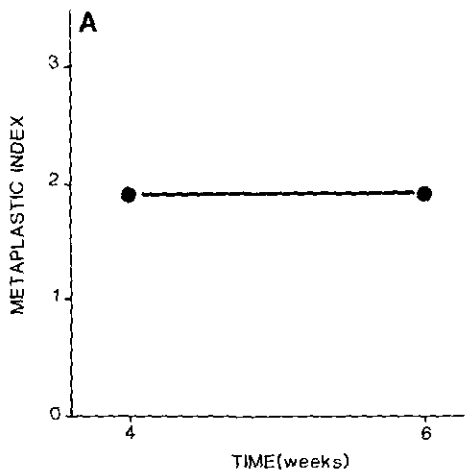
A number of studies have demonstrated the importance of vitamin A in maintaining the normal differentiation of tracheobronchial epithelium (*14,19–22*). In the absence of vitamin A, the mucociliary epithelium converts to squamous metaplasia, a lesion also observed after trauma (*23,24*) or exposure to toxic agents (*12,25*). After addition of chemical carcinogens, squamous metaplasia occurs in organ cultures of many types of epithelial cells (*26–28*). Both prevention and reversal of these lesions have been achieved after addition of retinoids to culture medium (*14,29–32*). Retinoids also appear effective in preventing the development and growth of chemically induced tumors in laboratory animals (*33–36*) although their mechanism(s) of action is unclear.

Asbestos is a physical carcinogen causing squamous metaplasia in the tracheobronchial epithelium (*8,10–12*) and is associated with an increased risk of bronchogenic carcinoma in man (*1*). Although chronic administration of the retinoid retinyl methyl ether prevents the appearance of asbestos-induced metaplasia in hamster tracheal organ cultures (*8*), the question of whether squamous metaplasia can be reversed after establishment of the lesion has received little attention. Accordingly, we addressed the questions: a) Can retinoids reverse preestablished, asbestos-associated squamous metaplasia? b) Does the time interval between the addition of asbestos and application of a retinoid affect the potential of the retinoid to reverse squamous metaplasia? and c) Can retinoids reverse squamous metaplasia induced by a chemical carcinogen such as BaP in the tracheal bioassay?

To determine the effects of retinoids on various treatment groups over extended time periods, it is of critical importance that time in culture has no effect on the development of metaplasia. Figure 2*A*, which is compiled from the pooled data of the 3 treatment groups (control, BaP, and asbestos), shows that length of time in culture from 4 to 6 weeks does not influence the extent of metaplasia. In contrast to the situation observed with asbestos, BaP does not cause a significant increase in squamous metaplasia in hamster tracheal organ cultures (Fig-

ure 2*B*). This observation supports our previous work (*37*) in which an increase in squamous metaplasia was found in cultures exposed to various concentrations of BaP over a 4-week period. Although metaplastic lesions were observed sporadically in the presence of BaP, these changes were not reproducible.

When data from all test groups is pooled, administration of RA results in a dosage-dependent decrease in the amount of squamous metaplasia at all time periods (Figure 3*A*). Additionally, all groups respond similarly to the retinoid, although the absolute amount of squamous metaplasia is more stiking, regardless of the concentration of retinoid, in the asbestos group as compared to control and BaP-exposed explants (Figure 3*B*).



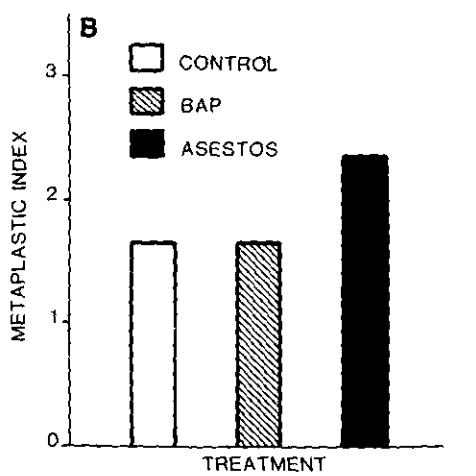
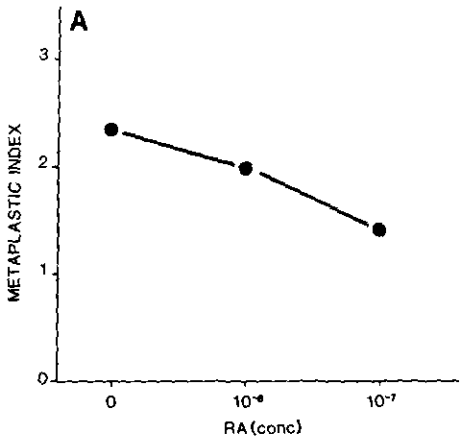


FIGURE 2. Effect of time in culture (*A*) and exposure to BaP or asbestos (*B*) on the development of squamous metaplasia in tracheal organ cultures. Time in culture (*A*) had no effect on the amount of metaplasia observed in the 3 test groups. Treatment of explants with asbestos (*B*) significantly increased the amount of metaplasia observed ($p < 0.001$), whereas exposure to BaP did not cause an increase in metaplasia in comparison to control explants. The dose of RA and treatment group (*A*) and dose of RA and time (*B*) were adjusted for in the statistical analyses.



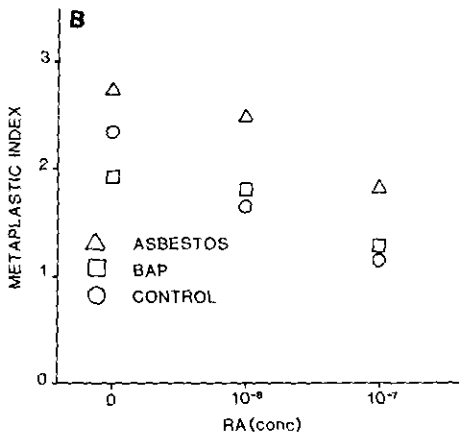


FIGURE 3. Effectiveness of RA in reversing squamous metaplasia in tracheal organ cultures. Under all circumstances, RA reversed squamous metaplasia in a dose-dependent manner ($p < 0.001$) (*A*), although the relative amount of squamous metaplasia was more striking in asbestos-exposed explants (*B*). Statistical analyses were adjusted for time and treatment in (*A*), whereas time alone was adjusted for in (*B*).

Figure 4 suggests that the effectiveness of RA decreases as the length of time between exposure to asbestos and initial application of RA increases. This observation is supported by the results of *in vivo* studies of others showing that delayed administration of retinoids leads to their diminished effectiveness in preventing mammary tumor growth (*38,39*). Unlike chemical carcinogens that are metabolized by tracheal epithelial cells, asbestos fibers are insoluble and remain entrapped in metaplastic lesions for 6 weeks and longer in culture. Thus, asbestos-induced squamous metaplasia appears to be more persistent and is reversed less effectively by retinoids than lesions associated with exposure to soluble toxicants.

Effects of Inhibitors of Polyamine Synthesis on Squamous Metaplasia

ODC is the first and rate limiting enzyme in the biosynthesis of polyamines (Figure 5), essential growth regula-

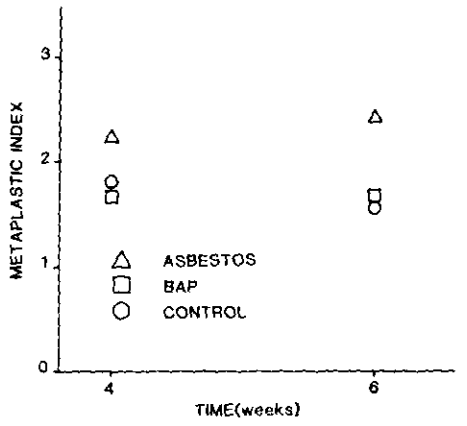


FIGURE 4. Effect of time on the efficacy of RA in reversing squamous metaplasia. Data suggest that RA is less effective in reversing asbestos-induced metaplasia when administered at 5 weeks in comparison to 3 weeks ($p < 0.09$).

tory molecules. The ability of phorbol compounds to induce ODC is related directly to their potency as tumor promoters, thus the induction of the enzyme is thought to be essential to the process of tumor promotion (*40*). In support of this hypothesis, DFMO, a specific, noncompetitive inhibitor of ODC, inhibits promotion in a number of experimental models including mouse skin (*41*), colon (*42*), pancreas (*43*), and mammary gland (*43*). Whereas DFMO induces differentiation of various tumor cell lines (*47,48*), it inhibits differentiation of preadipocytes (*49*) and myoblasts (*50*). RA also inhibits tumor promotion in mouse skin (*45*), ODC induction, levels of polyamines, and proliferation in mouse skin (*15*) and cultured cells (*46*).

To determine whether inhibitors of polyamine synthesis could inhibit squamous metaplasia in vitamin A-deficient tracheal organ cultures or explants exposed to crocidolite asbestos, we added DFMO, MGBG, a structural analog of spermidine and an inhibitor of *S*-adenosylmethionine decarboxylase (*51*) (Fig. 5), or DFMO followed by MGBG to culture media three times weekly. Under the latter circumstances, DFMO increases the uptake of MGBG by cells (*52*).

As shown in Figure 6, exposure to crocidolite asbestos or MGBG alone results in increased amounts of squamous metaplasia ($p < 0.05$) in comparison to controls. A higher percentage of explants exhibiting extensive squamous metaplasia is observed in the group exposed to crocidolite asbestos and MGBG, an observation supporting a possible additive effect of agents. In contrast, an increase in metaplasia was not observed in groups exposed to crocidolite with addition of DFMO, DFMO alone, or DFMO in combination with MGBG. Thus, DFMO appears to inhibit both asbestos- and MGBG-induced squamous metaplasia. Data provided in Figure 7 show no effects of DFMO on the development of metaplasia in vitamin A-deficient tracheal organ cultures, whereas MGBG alone or MGBG in combination with DFMO augment squamous metaplasia significantly ($p < 0.05$).

Table 1 illustrates the effects of various polyamines on DNA synthesis when added to the medium of tracheal organ cultures over a 3-week period. The addition of putrescine (1 and 10 mM) and spermidine (1 mM) cause significant ($p < 0.05$) increases in numbers of epithelial cells incorporating ^{3}H-thymidine, whereas spermine (1 mM) does not enhance the normal labeling index. Both spermine and spermidine were cytotoxic, as determined by histopathology, to tracheal epithelium at 10 mM (data not shown).

The data suggest that the polyamines, putrescine and spermidine, enhance epithelial cell replication and the development of squamous metaplasia in hamster tracheal organ cultures. Although increased proliferation of many eukaryotic cells has been observed after addition of putrescine and spermidine to monolayer cultures (*53,54*),

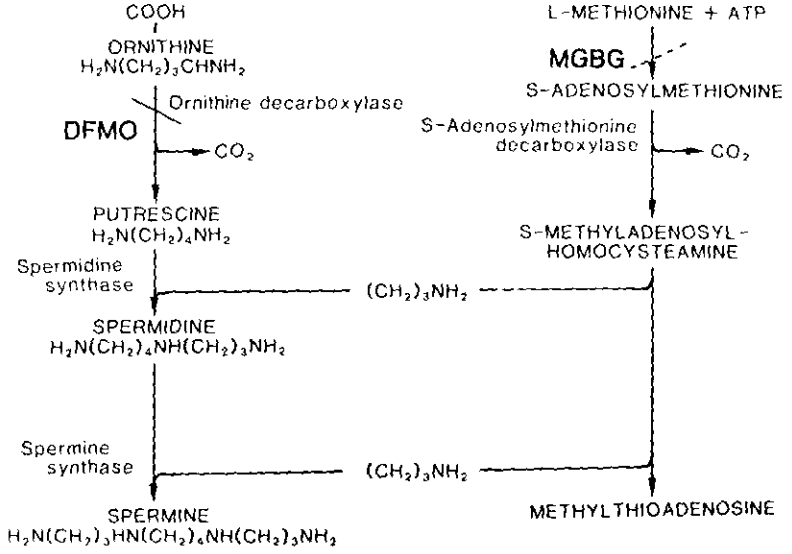


FIGURE 5. Diagram illustrating biosynthesis of polyamines.

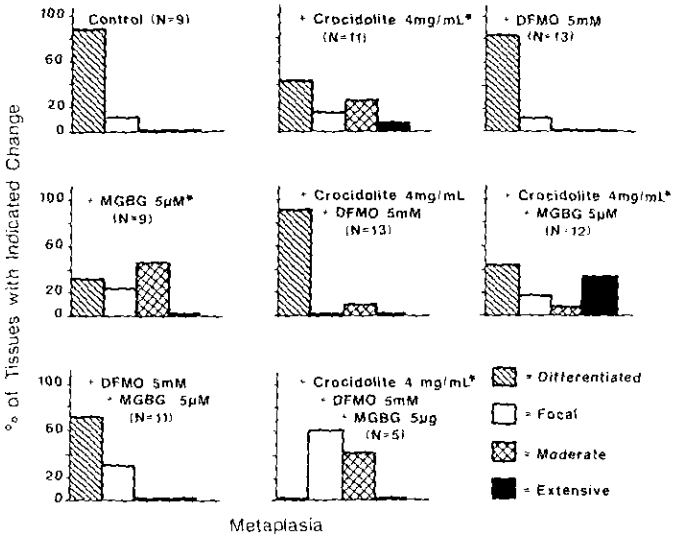


FIGURE 6. Effects of DFMO and MGBG on squamous metaplasia induced by asbestos. Addition of crocidolite (4 mg/mL) or MGBG (5 μM) caused an increase in squamous metaplasia in comparison to controls whereas no increase was observed with addition of crocidolite in combination with DFMO (5 mM). Asterisk (*) denotes $p < 0.05$.

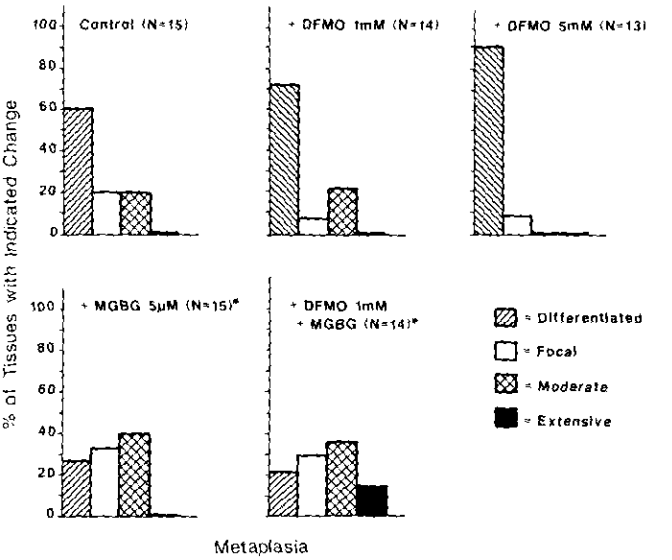


FIGURE 7. Effects of DFMO and MGBG on squamous metaplasia in tracheal organ cultures maintained in a complex medium encouraging squamous metaplasia. The addition of MGBG (5 μM) or DFMO (1 mM) in combination with MGBG caused a significant increase in squamous metaplasia in comparison to control cultures; asterisk (*) denotes $p < 0.05$.

Table 1. Incorporation of ^{3}H-thymidine in hamster tracheal epithelium after addition of polyamines.

Groups	n	% Labeled epithelial cells[a]
Control	19	2.42 ± 0.64
Putrescine, 10 mM	14	7.28 ± 1.80[b]
Putrescine, 1 mM	19	6.96 ± 1.31[b]
Spermine, 1 mM	16	2.36 ± 0.69
Spermidine, 1 mM	15	6.95 ± 0.68[b]

[a]Mean ± SE, 100 epithelial cells from each of five serial sections were counted for each explant.

[b]Increased in comparison to untreated controls ($p < 0.05$).

the finding that these polyamines augment basal cell hyperplasia and metaplastic differentiation of tracheal epithelial explants is novel. The inhibition of asbestos-induced squamous metaplasia by DFMO, a drug depleting *de novo* synthesis of all polyamines (*55*), further strengthens the hypothesis that polyamines are critically involved in the induction of squamous metaplasia by asbestos. For reasons that are unclear, DFMO did not appear to inhibit the squamous metaplasia observed with vitamin A deficiency or vitamin A deficiency in combination with MGBG (Fig. 7). In comparison to putrescine and spermidine, spermine appears to be relatively less important in cellular proliferation as growth inhibition only occurs when intracellular pools decline to ≤ 60% of normal (*55*). However, this polyamine appears to play an important physiological role in intracellular calcium homeostasis (*56*). Although MGBG can block synthesis of both spermine and spermidine, it also increases cellular transport of extracellular polyamines, even in the presence of DFMO (*51*). Thus, increased accumulation of putrescine and/or spermidine under these circumstances might explain the enhanced amount of squamous metaplasia observed in MGBG-treated explants.

In conclusion, depletion of polyamines by DFMO or treatment with retinoids appear to be an effective means of preventing and/or reversing asbestos-associated squamous metaplasia *in vitro*. The use of these agents may be rewarding as prophylactic or therapeutic approaches to lung disease in man.

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